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John Negrey  
*San Jose State University*

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THE EFFECT OF DIETARY METHYLMERCURY ON  $\text{Na}^+, \text{K}^+$ -ATPASE ACTIVITY  
AND GROWTH IN FALL-RUN CHINOOK SALMON (*O. TSHAWYTSCHA*) FROM  
CALIFORNIA'S CENTRAL VALLEY

A Thesis

Presented to

The Faculty of Moss Landing Marine Laboratories

San José State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

John Patrick Negrey

December 2013

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The Designated Thesis Committee Approves the Thesis Titled

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AND GROWTH IN FALL-RUN CHINOOK SALMON (*O. TSHAWYTSCHA*) FROM  
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By

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December 2013

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## ABSTRACT

### THE EFFECT OF DIETARY METHYLMERCURY ON $\text{Na}^+,\text{K}^+$ -ATPASE ACTIVITY AND GROWTH IN FALL-RUN CHINOOK SALMON (*O. TSHAWYTSCHA*) FROM CALIFORNIA'S CENTRAL VALLEY

by John P. Negrey

Hatchery reared Chinook salmon from California's Central Valley were fed for 67 days (Apr-Jun, 2008) on fish pellets mixed with either 0, 1, 3, or 5  $\mu\text{g}\cdot\text{g}^{-1}$  methylmercury hydroxide. Weight, fork length, condition factor, and  $\text{Na}^+,\text{K}^+$ -ATPase measurements were determined every two weeks and a 96-h seawater challenge was conducted at the conclusion of the experiment.

Results from two-way ANOVA, with treatment and date as independent variables, indicated no significant differences for weight ( $F_{3,32} = 1.38$ ;  $P = 0.280$ ), length ( $F_{3,32} = 0.986$ ;  $P = 0.412$ ) and condition factor ( $F_{3,32} = 0.239$ ;  $P = 0.869$ ). Post-hoc analysis following two-way ANOVA indicated mean ATPase activity in the high ( $\bar{x} = 3.08$ , S.E. = 0.19;  $P = 0.008$ ) and medium treatments ( $\bar{x} = 2.86$ , S.E. = 0.57;  $P = 0.017$ ) was significantly increased in early May compared with the control group ( $\bar{x} = 1.47$ , S.E. = 0.34). The results from the 96 h seawater challenge were consistent with those of other studies indicating weight has the greatest influence for survival in the transition from freshwater to seawater. Overall, results from this study indicated methylmercury, a known neurotoxin, altered ATPase activity in fall-run Chinook but did not significantly affect mortality in the transition from freshwater to seawater.

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## Table of Contents

1.0 Introduction.....	1
2.0 Methods.....	6
2.1 Experimental Design.....	6
2.2 Methylmercury Exposure.....	8
2.3 Gill Filament Collection and Growth Measurements .....	9
2.4 Measurement of Na <sup>+</sup> ,K <sup>+</sup> -ATPase Activity and Mercury.....	11
2.5 Seawater Adaptability Tests.....	13
2.6 Data Analysis .....	13
3.0 Results.....	14
3.1 Mercury Accumulation and Growth .....	14
3.2 Na <sup>+</sup> ,K <sup>+</sup> -ATPase Activity .....	17
3.3 96-h Seawater Adaptability Test .....	20
4.0 Discussion.....	25
5.0 Conclusion .....	30
References.....	32

## List of Tables

Table 1	Fork length, weight, and condition factor (K) post seawater challenge in control and treatments fed dietary methylmercury .....24
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## List of Figures

Figure 1	Total mercury concentrations in axial tissue of juvenile Chinook salmon .....	16
Figure 2	Whole body versus axial tissue mercury .....	17
Figure 3	Mean fork length (a), weight (b), and condition factor (c) .....	18
Figure 4	Gill $\text{Na}^+, \text{K}^+$ -ATPase activity .....	19
Figure 5	Average daily rate of change in gill $\text{Na}^+, \text{K}^+$ -ATPase activity .....	21
Figure 6	Cumulative survival curves for Chinook salmon during a 96-h seawater adaptability test .....	22
Figure 7	$\text{Na}^+, \text{K}^+$ -ATPase activity pre and post 96-h seawater exposure .....	23

## 1.0 Introduction

The burning of fossil fuels and poor mining practices of the 18<sup>th</sup> and 19<sup>th</sup> centuries created a legacy of mercury (Hg) contamination in modern day ocean, atmosphere, and terrestrial systems (Mason et al., 1994; Selin, 2009). As much as 70% of the atmospheric Hg in the past 100 years has been generated from anthropogenic sources (Schuster, 2002). This has greatly increased the risk of exposure for many marine and freshwater organisms. Comparisons of Hg concentrations in modern and preindustrial teeth of Arctic marine mammals (walrus, beluga, and ring seals) have increased as much as 17 times in the last few centuries (Outridge et al., 2002; 2009). Similarly, feathers and hair from other Arctic animals significantly increased in Hg concentrations, despite an absence from any major point source of Hg (Dietz et al., 2009).

Mercury is naturally occurring in the environment and found in many different forms. The inorganic and elemental form,  $\text{Hg}^0$ , is pure liquid metal and rarely found in the environment, but it can persist from 6 months to 2 years in the atmosphere as a vapor (Lindqvist and Rodhe, 1985; Pai et al., 1998). Mercury can be bound to other compounds in a monovalent (as  $\text{Hg}[\text{I}]$ ) or divalent state ( $\text{Hg}[\text{II}]$ ). These forms generally have short residence times in the atmosphere (hours to days) and typically are removed within tens to hundreds of kilometers from their source (Schroeder and Munthe, 1998). As a result, atmospheric Hg fluxes to the terrestrial environment often exceed typical global background depositional rates (Mason et al., 1994; Schroeder and Munthe, 1998).

In California and other regions of the world, historic mining of cinnabar-ore (HgS) has greatly increased Hg flux to local watersheds. Most Hg mining occurred during the latter part of the 18<sup>th</sup> century and early part of the 19<sup>th</sup> century when Hg was used for gold (Au) mining, industrial, and agricultural applications (Hylander and Meili, 2005). In California, runoff and weathering from the abandonment of Au and Hg mines have resulted in its transport to many of the state's lakes, rivers, streams, and surrounding wetlands (Wiener and Suchanek, 2008) where significant reservoirs of Hg in sediments persist. Thus, the potential for Hg released into these environments is still great, even in the absence of mining.

The biologically relevant and neurotoxic form of Hg is methylmercury (MeHg). In the atmosphere, MeHg or dimethylHg are expected to be short lived (Niki et al., 1983). However, there is recent evidence that indicates MeHg may be longer lived in fog water and could be a considerable input to certain coastal environments (Weiss-Penzias et al., 2012). The formation of MeHg, from Hg(II), is typically mediated by strains of sulfate- and iron-reducing bacteria living in aquatic systems (Benoit et al., 2001; Kerin et al., 2006; Ullrich et al., 2001). Once formed, the biologically available MeHg can enter into the aquatic food chain. The subsequent biomagnification from bacteria to plankton to fish is generally orders of magnitude greater than the surrounding water and increased with every trophic step (Wiener et al., 2002).

There are considerable health risks for Hg exposure. In humans, Hg adversely affects the central nervous system and the developing brain. It inhibits myosin ATPase activity

(Moreira et al., 2003) and can cause oxidative stress via glutathione (GSH) depletion, thus increasing reactive oxygen species, which leads to apoptosis of the cell (Park and Park, 2007; Reus et al., 2003). In fish, which are most susceptible to Hg, oxidative stress responses can occur under waterborne and dietary exposure (Berntssen et al., 2003; Huang et al., 2010). Additionally, Hg can impair foraging efficiency, reproduction, and cause disruptions in the functioning of the endocrine system (Drevnick and Sandheinrich, 2003; Hammerschmidt et al., 2002). The physiological impacts Hg has on fish species is cause for alarm. This is especially true for fish species, such as Chinook salmon (*Oncorhynchus tshawytscha*), which play a critical role in the ecology of a watershed and which we, as humans, rely upon as a food source.

Spawning grounds of the Chinook are distributed in rivers and streams along the northern Pacific Rim from Japan to central California (Healey, 1991). In the United States, the National Oceanic and Atmospheric Administration (NOAA) has identified 17 evolutionary significant units (ESUs) or distinct populations of Chinook salmon along the Pacific Northwest (Washington, Oregon, Idaho, and California). In California's Central Valley, comprised of the Sacramento-San Joaquin River System (SSJR), the fall and late-fall run of Chinook salmon historically had runs of a million or more fish until the earlier part of the 19<sup>th</sup> century (Healey, 1991). Habitat loss, over fishing, and the degradation of water quality severely affected these populations, some to the brink of extinction (Fisher, 1994; Yoshiyama et al., 1998). As of 2012, both Central Valley fall- and late fall-run Chinook salmon are listed as a "species of concern" by the United States Fish and

Wildlife Service (USFWS) under the Endangered Species Act (ESA). The Central Valley spring-run ESU of Chinook salmon is listed under the ESA as threatened.

The ontogenetic migration of Chinook salmon is part of a life cycle that begins and ends in freshwater. Newly hatched salmon, or alevins, spend several weeks hidden in a gravel nest feeding off an attached yolk sac. Once they are large enough, the salmon, now fry, leave the safety of their nest to forage and feed on their own. After a short period, the fry develop distinct fingerling markings on the sides of their body and enter the parr stage. Once large enough and environmental conditions are favorable, they begin making their journey to the sea. At this stage, the parr have become smolts and must prepare themselves by adjusting their bodies and metabolism to handle the new hypertonic environment. Such changes include the loss of the vertical parr markings, silvering of the body, and an increase in gill  $\text{Na}^+, \text{K}^+$ -ATPase activity. Adult Chinook will spend three years on average in the Pacific Ocean feeding and growing until the time is right to return to the river and complete the life cycle.

In the SSJR there are four spawning “runs” of Chinook that exhibit two different types of behaviors, either stream or ocean-type. The major difference between types being the time the smolts spent in freshwater before heading to sea. Spring-run Chinook enter the rivers between May and June and delay spawning until late August or September. Juveniles from spring-run adults exhibit stream-type behavior staying in the system upwards to a year. Winter-run Chinook tend to overlap with spring-run fish but spawn earlier (May-June). The winter-run is unique to the SSJR. Chinook from this run

can exhibit both stream and ocean-type behavior. The fall and late-fall run Chinook enter the SSJR between September and October, spawn between December and January (Healey, 1991), and comprise over 90% of the SSJR population. Chinook in these runs exhibit ocean-type behavior with juveniles spending as little as a few months in fresh water before migrating to sea.

In the SSJR, juvenile Chinook salmon actively rear in many of the wetland habitats associated with the state's Coastal Range Hg and Au mining region (Sommer et al., 2001). Wetlands enhance production of MeHg (Hurley et al., 1995; Rudd, 1995) and greater growth rates occur in Chinook salmon utilizing these wetlands as opposed to the main stem of the river (Sommer et al., 2001). This indicates there may be an increased risk of MeHg accumulation. In silversides (*Menidia beryllina*), which are similar in size and similar in foraging ecology as the juvenile Chinook, dry weight Hg tissue concentrations were consistently increased in sites exposed to major wetland's flows within the SSJR (Slotton et al., 2002). Transitioning from a hypotonic to hypertonic solution requires a significant change in the physiology of the Chinook salmon (smoltification) and represents the chemical, physical, and behavioral changes necessary to prepare the salmon for life at sea (Hoar, 1988).

The purpose of this study was to determine the effect that dietary MeHg may have on  $\text{Na}^+, \text{K}^+$ -ATPase activity in juvenile Chinook salmon during this critical transformation. ATPase activity is one of many metabolic characteristics of smoltification in salmon. Zaugg and McLain (1970) were the first to demonstrate an increase in gill ATPase activity

during the parr-smolt transformation of Coho salmon. Subsequently, measuring changes in ATPase activity has become standard practice when evaluating the effects of pollutants on salmon smoltification. A 96-h seawater adaptability test was used to determine whether dietary MeHg reduced survival during the transition from freshwater to seawater. Morphometric measurements (length and weight) were collected throughout the experiment to determine whether dietary MeHg adversely affected growth.

## **2.0 Methods**

### *2.1 Experimental Design*

All protocols established for the care and proper handling and treatment of all animals used in this experiment followed protocols approved by San José State University's Institutional Animal Care and Use Committee (IACUC Protocol #902). Experiments were conducted in the spring of 2008 at Moss Landing Marine Laboratories (MLML) to assess the effects dietary MeHg had on ATPase activity, growth, and seawater adaptability in hatchery reared late fall-run Chinook salmon. The hatchery-reared salmon used in this study were representative of the native Central Valley fall and late-fall run Chinook salmon. Using hatchery reared fall-run Chinook salmon had the least amount of impact on the native population while providing a greater number of individuals for use in the experiment than would have been achieved through field collections of wild Chinook salmon. Nimbus Fish Hatchery, operated by the California Department of Fish and Wildlife (CDFW), located in Rancho Cordova, California, provided all of the salmon for the study.

Chinook salmon were transported by truck from the fish hatchery to Moss Landing Marine Laboratories in a closed aerated circular tank. During transport (approx. 3 h), temperature was checked periodically and maintained at  $\sim 12^{\circ}\text{C}$  by the addition of ice to the tank. This method of transportation was approved by hatchery personnel and had been successfully used in the past for obtaining salmon from the hatchery. Upon arrival at Moss Landing Marine Laboratories, the fish were immediately transferred to an 1100-L semi-closed freshwater system located in the Moss Landing Marine Laboratories aquarium room.

After 7 days of acclimation, salmon were randomly sorted, five at a time, into 12 “cages” within aquaria. Each cage was then randomly assigned one of four diets (control, low, medium, or high). The cages consisted of a nylon mesh bag (1 mm diameter mesh) with a PVC frame insert to give support and measured approximately 55x25x46 cm. Each cage was housed in a larger freshwater filled tank measuring approximately 180x60x36 cm. Two separate tanks were needed to house all 12 cages for the experiment. The tanks were adjacent to one another, on the same water filtration system, and identical in every aspect.

The freshwater used in the experiment came from the local municipal water source and was pre-filtered through activated carbon and UV sterilized. Water temperature was monitored daily and held consistently at  $14^{\circ} \pm 1^{\circ}\text{C}$  for the duration of the experiment and water was tested, at a minimum of once per week, for nitrate, ammonia, chlorine, and pH using a standard over-the-counter aquarium test kit. To remove nitrates and to avoid



large water changes, freshwater was added to the system via header tank continuously at rate of  $20 \text{ mL min}^{-1}$ . The incoming water was first mechanically filtered, then filtered through activated carbon, and finally treated with AquaChem chlorine remover before entering the system. The entire system housing the experiment consisted of one large water table positioned above the two tanks containing the cages. Freshwater drained from the water table through two separate (one for each tank) PVC tubes filled with physical media (Bio Balls) that contained nitrogen-reducing bacteria. Freshwater from both tanks was then pumped through a three-stage filter system and returned to the water table.

## *2.2 Methylmercury Exposure*

The dietary levels in this study were chosen to achieve a range of previously reported Hg tissue concentrations in juvenile Chinook (Henery et al., 2010) and other small fish species collected in the SSJR (Ackerman and Eagles-Smith, 2010). Methylmercury contaminated diets were prepared by mixing fish pellets (BioOregon) with ethanol containing mono-methyl-mercuric hydroxide ( $100 \text{ } \mu\text{g CH}_3\text{Hg-OH mL}^{-1}$ , referred to as MeHg from here on) obtained from Brooks-Rand. Juvenile Chinook salmon were fed a contaminated diet with one of four concentrations of MeHg: control (ethanol only), low ( $1 \text{ } \mu\text{g MeHg g}^{-1}$ ), medium ( $3 \text{ } \mu\text{g MeHg g}^{-1}$ ), or high ( $5 \text{ } \mu\text{g MeHg g}^{-1}$ ). Salmon were fed daily to satiation from April 10 thru June 16, 2008 (67 d).

The MeHg/ethanol mixture for the low MeHg diet was prepared by mixing 1 mL MeHg and 59 mL ethanol to yield  $1.67 \text{ } \mu\text{g MeHg mL}^{-1}$ . Three milliliters of the  $1.67 \text{ } \mu\text{g}$

MeHg mL<sup>-1</sup> solution was added to 5 g of pellets (Yield 5 µg MeHg 5 g<sup>-1</sup> of pellets = 1 µg MeHg g<sup>-1</sup>). These volumes allowed 15 diets to be prepared at a time for each treatment minimizing handling and exposure of researchers to MeHg. The medium MeHg diets were prepared by mixing 3 mL MeHg and 57 mL ethanol to yield 5 µg MeHg mL<sup>-1</sup> (by adding 3 mL to 5g of pellets = 3 µg MeHg g<sup>-1</sup>). The high MeHg diets were prepared by mixing 5 mL MeHg and 55 mL ethanol to yield 8.33 µg MeHg mL<sup>-1</sup> (adding 3 mL to 5g of pellets = 5 µg MeHg g<sup>-1</sup>). Final concentrations of MeHg were determined in each of the treatments (mean ± S.D., n = 3): 0.05 ± 0.002 (control), 1.35 ± 0.05 (low), 2.47 ± 0.14 (medium), and 4.21 ± 0.29 (high) µg MeHg g<sup>-1</sup>.

The appropriate MeHg/ethanol mixture was dispensed over the pellets and mixed using a pipette tip. The control diet was treated with ethanol only. The pellets were mixed again after 24 and 48 h, and the control (ethanol only) pellets were checked to ensure there was no vapor still emanating from them. Pellets were stored in clean glass vials and kept in a cool dark room until use.

### *2.3 Gill Filament Collection and Growth Measurements*

Primary gill filaments were extracted from each fish for Na<sup>+</sup>,K<sup>+</sup>-ATPase analysis according to the method described in McCormick (1993) and whole body fish samples were preserved for Hg analysis. Total and fork length measurements were also recorded, in millimeters, using a standard tape ruler and individual weights were measured using an AWS digital pocket scale calibrated to ± 0.1 g. Using these measurements, a condition

factor ( $K$ ) was calculated based on the formula  $K = (10^5 \times W) / L^3$ ; where  $W$  is the mass of fish in grams and  $L$  is the (caudal) fork length in millimeters.

Ten juvenile salmon obtained from the hatchery were initially sampled for length, weight, and  $\text{Na}^+$ ,  $\text{K}^+$  ATPase activity in mid-March (2008) before the start of the experiment. Length and weight measurements, for individual fish, coincided with gill extraction to minimize handling of the fish during the course of the experiment. Every two weeks from April 10<sup>th</sup>, 2008 through June 16<sup>th</sup>, 2008, four fish were netted and removed from each treatment and placed in a small cooler containing a pre-mixed solution of tricaine methanesulfonate (MS-222) and ambient tank water. Fish were held in the cooler containing the pre-mixed solution until a loss of righting reflex was observed (~3 min) indicating the fish had become suitably anesthetized. Once anesthetized, fish were removed from the pre-mixed MS-222 solution and “dipped” into small cooler containing ambient tank water. This was done to minimize the exposure of MS-222 to any researchers handling the anesthetized fish.

Salmon were placed on a clean Teflon® coated board and length measurements were recorded. Salmon were weighed then returned to the Teflon board for gill filament extraction. A small set of tweezers were used to pull back the operculum, exposing the primary gill filaments, and a pair of sharp-pointed dissecting scissors were used to excise the gill filaments (typically 5-7 filaments cut to half their length). The filaments were placed in a one-milliliter micro centrifuge tube containing a sucrose,  $\text{Na}_2\text{EDTA}$ , and imidazole (SEI) solution and stored at  $-80^\circ \text{C}$ . The salmon recovered in a cooler

containing ambient tank water and placed back into their respective treatment upon completion of the gill filament extraction. If at any time during the course of the experiment, a salmon exhibited any indication of suffering, the individual fish was removed and euthanized by overdose of MS-222.

#### *2.4 Measurement of $\text{Na}^+, \text{K}^+$ -ATPase Activity and Mercury*

The method for analysis of  $\text{Na}^+, \text{K}^+$ -ATPase activity followed a more detailed version of the published method by McCormick (1993). Assay mixtures were made just before analysis. Gill filament samples were thawed immediately prior and kept on ice throughout the analysis. Samples were tested in triplicate and activity calculated as the difference in ATP hydrolysis in the absence and presence of ouabain. Activity was normalized for protein content using a protein assay reagent kit (Pierce) on remaining sample homogenate. Final activity was expressed as micromoles of ADP per milligram of protein per hour. All samples were tested on a Thermomax 96-well plate reader.

Tissue samples were analyzed for total Hg by modified United States Environmental Protection Agency (USEPA) method 7473. In general, nearly all tissue Hg in fish (>95%) is considered MeHg (Bloom, 1992). However, the percentage of methyl to total Hg in tissue can vary temporally within and among different species (Mason et al., 2000). In this study, total Hg tissue concentrations were considered MeHg. All fish were dissected and homogenized using standard clean procedures and were analyzed on a Milestone Direct Mercury Analyzer (DMA-80). Briefly, the method involves a drying

step followed by combustion, purging, trapping on gold, desorption, and atomic absorption detection.

Quality control (QC) samples included blanks, duplicate samples, and certified reference material (CRM). A validation of the standard curve for the DMA-80 was made daily using known standards and a new calibration curve was generated every two weeks. Salmon from each dietary treatment were randomly selected for analysis. All fish samples for this study were analyzed as individuals either whole-body or on an axial-tissue wet weight (ww) basis. Chinook salmon were measured for MeHg accumulation in each of the dietary treatments (3-10 fish per treatment with 12 treatments) at the conclusion of the experiment to determine the effectiveness of the dosing.

To determine whether axial muscle tissue adequately reflected whole body MeHg concentrations, 14 fish were randomly selected, homogenized and then analyzed whole body for MeHg. Results from this analysis were compared with mean MeHg concentrations in axial tissue from each dietary type. Assimilation efficiency or “uptake” of the dietary MeHg administered to all the salmon over the course of the experiment also was estimated. To do so, the mean MeHg concentration of each dietary treatment was multiplied by each individual fish’s weight not analyzed for MeHg (this did not include control treatments). Hence, when MeHg data were available, it was used, and where there were no MeHg tissue data available, the mean MeHg concentration of the treatment was multiplied by the individual fish weight to give an estimate of whole body MeHg concentration.

## *2.5 Seawater Adaptability Tests*

Just prior to the conclusion of the study, each treatment underwent a 96 h seawater challenge to measure the success of smoltification (Komourdjian et al., 1976; Saunders et al., 1985). Filtered seawater from Monterey Bay, California was added to the tanks housing the treatments until the entire system was at ambient SW conditions (approximately 4 hours to achieve ~33 ppt). Survivability was monitored at 2, 6, 12, 24, and 12-hour intervals thereafter to the 96th hour in each of the treatments. During the seawater challenge if a fish exhibited loss of righting reflex the fish was removed from the experiment and euthanized by overdose of MS-222. At each time interval, fish were removed and euthanized if necessary and the appropriate samples and measurements were collected. At the conclusion of the seawater challenge (June 20, 2008; 67 d), remaining fish in the study were measured for total and fork length, weighed, and gill biopsied. Salmon were then euthanized via overdose MS-222 ( $> 1 \text{ g L}^{-1}$ ), individually wrapped in aluminum foil, placed in a labeled Ziploc bag, and either preserved at  $-20^{\circ} \text{ C}$  or flash frozen in Cryo Tubes® for further study .

## *2.6 Data Analysis*

Statistics and random number generation were performed using SPSS for Windows version 16.0 (SPSS Inc., Chicago, IL, USA). ATPase data was processed in MATLAB Student version R2007a. Analysis of variance tests were used to assess differences in  $\text{Na}^{+}, \text{K}^{+}$ -ATPase activity, condition factor, length, and weight among treatments. Condition factor, weight, and length were examined as potential covariates using ordinary least squares regression (OLS).

Alpha levels were set at 0.05 for all analysis of variance tests. Data were checked for normality using the residuals in regression analysis or a K-S test in analysis of variance tests. Studentized residuals were used to check for homogeneity of variance and Cook's distance was used to check for outliers in the data. A Levene's test was used to assess the equality of variances and regression coefficients ( $\beta$ ) were used to test the assumption of homogeneity of regression slopes in analysis of covariance.

A Cox proportional hazards test was used to evaluate the effects of treatment and the influence of covariates on survival times in the 96 h seawater challenge. A Cox proportional hazards model is analogous to a multiple linear regression model with the exception that the dependent variable is related to an event, in this case survival, and there are only two possible outcomes: alive or dead. The Cox hazards model assumes that the proportional hazards stay constant over time, that is, the baseline hazard for each treatment does not change. This assumption was tested using log-log plots and a goodness of fit test using the Schoenfeld residuals (Harrell et al., 1996; Kleinbaum and Klein, 2012).

### **3.0 Results**

#### *3.1 Mercury Accumulation and Growth*

All Hg values reported are on a wet weight basis (ww). The percentage moisture for food pellets (control-ethanol only) administered in this study was  $5.8 \pm 0.2\%$ .

Approximately 1.5 grams of axial muscle tissue were randomly selected from one salmon

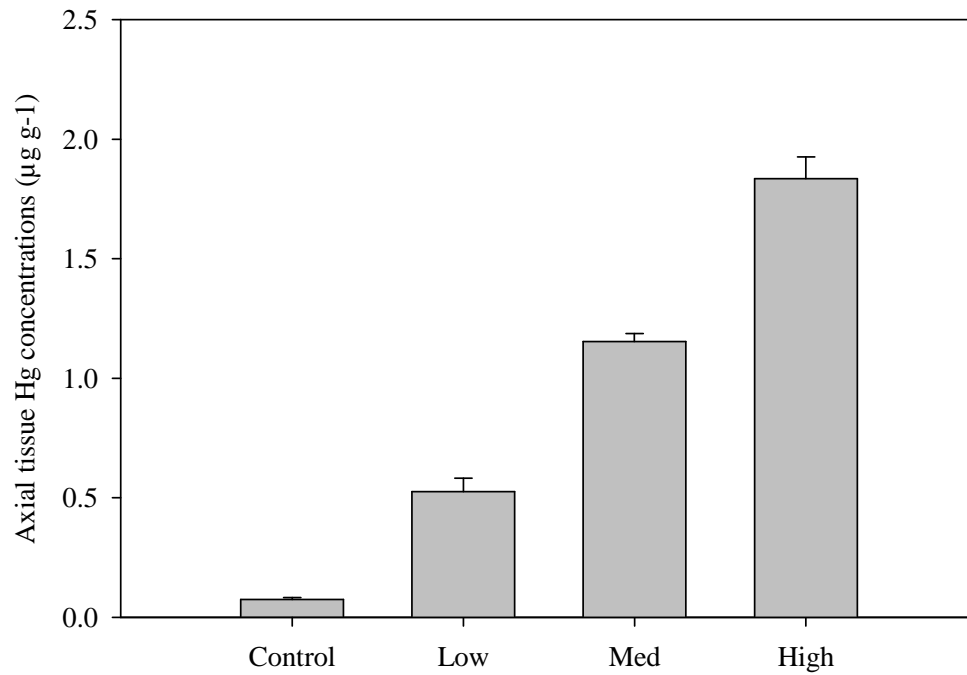
in each treatment ( $n = 12$ ) to calculate the average percentage moisture for all the salmon in the study which was  $78.3 \pm 0.2\%$ .

Mean MeHg concentrations and standard error were calculated for each of the dietary treatments (3 replicates/treatment; Figure 1). As expected, the control individuals fed the uncontaminated diet had little to no MeHg in axial muscle tissue ( $0.075 \pm 0.04 \mu\text{g MeHg g}^{-1} \text{ ww}$ ). Methylmercury concentrations in the low ( $0.53 \pm 0.06 \mu\text{g MeHg g}^{-1} \text{ ww}$ ), medium ( $1.15 \pm 0.03 \mu\text{g MeHg g}^{-1} \text{ ww}$ ), and high treatments ( $1.83 \pm 0.09 \mu\text{g MeHg g}^{-1} \text{ ww}$ ) were roughly half the concentration of the dietary MeHg that was being administered to each treatment.

Whole body MeHg concentrations were consistent with axial muscle MeHg concentrations (Figure 2). The relative percentage difference between whole and axial muscle tissue MeHg was 14%. Based on the total amount of MeHg administered ( $2700 \mu\text{g}$ ) 82% was assimilated into tissues during the 67 d period for all dietary treatments.

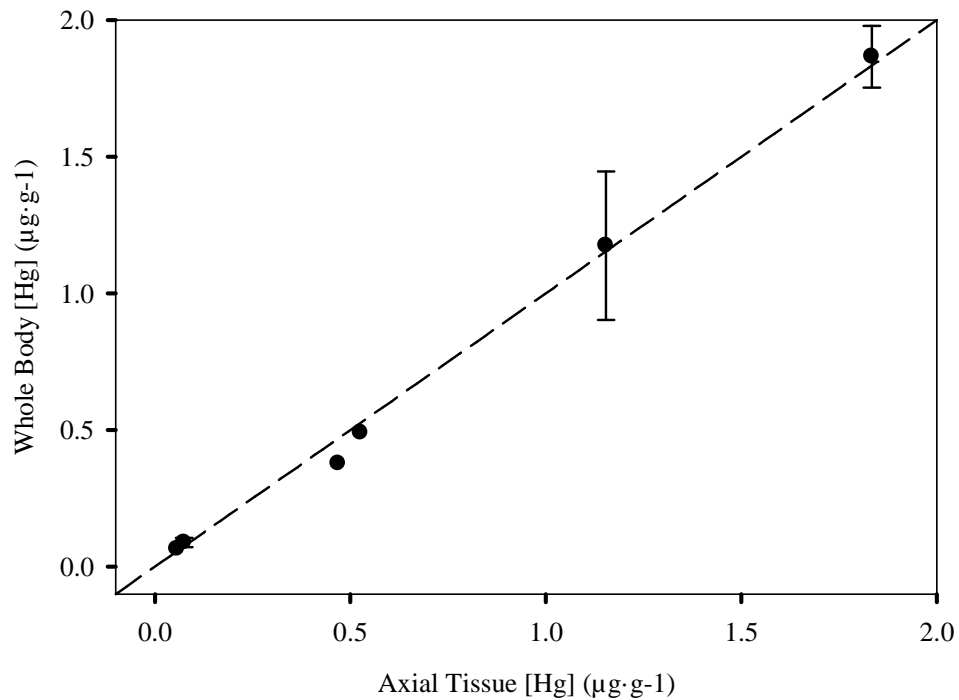
Weight and fork length increased from March until June in all treatments (Figure 3a and b). Results from a two-way ANOVA indicated there was a significant effect of date on weight ( $F_{3, 32} = 76.6$ ;  $P < 0.001$ ) but not treatment ( $F_{3, 32} = 1.38$ ;  $P = 0.280$ ). Similarly, results from a two-way ANOVA indicated there was a significant effect of date on fork length ( $F_{3, 32} = 74.87$ ;  $P < 0.001$ ) but not treatment ( $F_{3, 32} = 0.986$ ;  $P = 0.412$ ). There was no statistically significant interaction between date and treatment for either weight ( $F_{9, 32} = 0.967$ ;  $P = 0.484$ ) or length ( $F_{9, 32} = 1.01$ ;  $P = 0.452$ ).





**Figure 1.** Total mercury concentrations in axial tissue of juvenile Chinook salmon after 67 days fed a dietary food mixture containing no (control), 1 (low), 3 (med), or 5 (high)  $\mu\text{g}\cdot\text{g}^{-1}$  methylmercury hydroxide ( $\text{CH}_3\text{HgOH}$ ) from April 10 thru June 14. Error bars are mean  $\pm$  1 S.E.M.

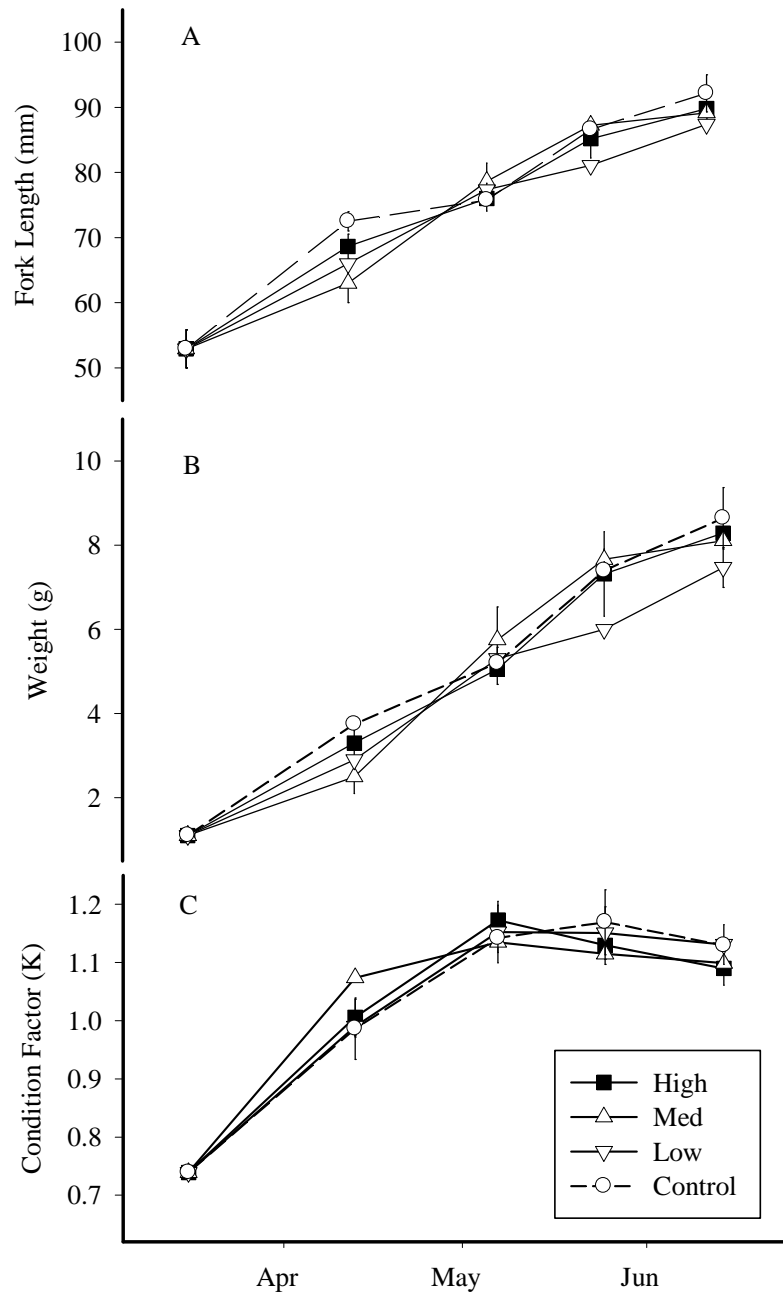
The condition factor (Figure 3c) increased steadily from March until early May and remained constant through June. The results from a two-way ANOVA indicated that there was a significant effect of date ( $F_{3, 32} = 6.97$ ;  $P < 0.001$ ) but not treatment ( $F_{3, 32} = 0.239$ ;  $P = 0.869$ ) on condition factor. There was no statistically significant interaction between date and treatment for condition factor ( $F_{9, 32} = 0.513$ ;  $P = 0.854$ ).



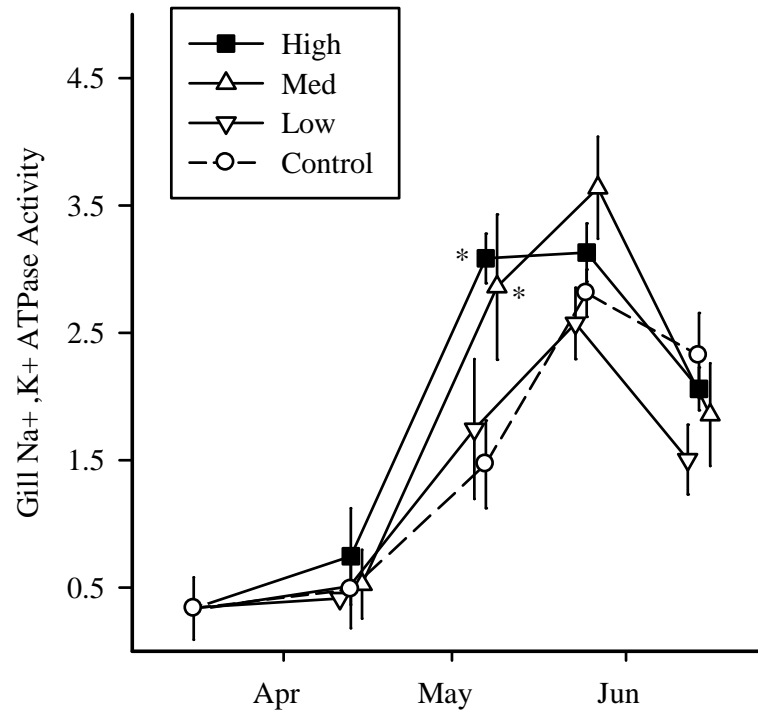
**Figure 2.** Whole body versus axial tissue mercury in Chinook salmon. Dashed line is 1:1 ratio between whole body and axial tissue mercury.

### 3.2 $\text{Na}^+, \text{K}^+$ -ATPase Activity

Gill  $\text{Na}^+, \text{K}^+$ -ATPase activity increased in all treatments from March until a peak in May followed by a decline in activity in June (Figure 4). Two models were used to evaluate changes in ATPase activity by date and treatment. In the first model, results from a two-way ANOVA indicated there was a significant effect of date ( $F_{3, 28} = 25.85$ ;  $P < 0.001$ ) and treatment ( $F_{3, 28} = 3.15$ ;  $P = 0.041$ ) on ATPase activity. There was no statistically significant interaction between date and treatment ( $F_{9, 28} = 1.373$ ;  $P = 0.247$ ).



**Figure 3.** Mean fork length (a), weight (b), and condition factor (c) for juvenile Chinook salmon. There were no significant differences ( $P < 0.05$ ) from the control group. Error bars are mean  $\pm$  1 S.E.M.



**Figure 4.** Gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity (μmol ADP mg protein<sup>-1</sup> h<sup>-1</sup>) from March 16 thru June 14. Date values are offset from controls for visual purposes. \*Values differ significantly ( $P < 0.05$ ) from the control group. Error bars are  $\pm 1$  S.E.M.

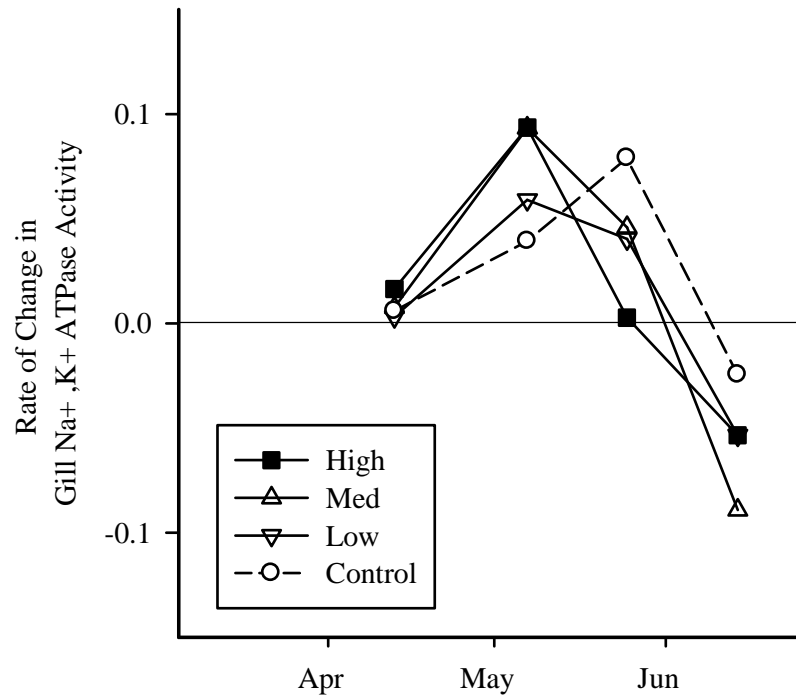
Post hoc analysis (versus control), using Holm-Sidak method, indicated significant differences between treatment means sampled on a common date. Mean ATPase activity (μmol ADP mg protein<sup>-1</sup> h<sup>-1</sup>) in the high ( $\bar{x} = 3.08$ , S.E. = 0.19;  $P = 0.008$ ) and medium treatments ( $\bar{x} = 2.86$ , S.E. = 0.57;  $P = 0.017$ ) was significantly greater in early May over activity in the low treatment ( $\bar{x} = 1.74$ , S.E. = 0.55;  $P = 0.577$ ) as compared to the control diet ( $\bar{x} = 1.47$ , S.E. = 0.34).

In order to evaluate the apparent enhanced ATPase activity in salmon fed greater MeHg diets a second statistical model employing a covariate was included to account for differences in ATPase activity because of fish size. Fork length, mass, and condition factor were regressed independently against ATPase activity to determine which variable would be the best covariate. Results from ordinary least squares (OLS) regression indicated mass ( $R^2 = 0.42$ ;  $P < 0.01$ ) explained the most variability in ATPase activity between April and June followed by fork length ( $R^2 = 0.39$ ;  $P < 0.01$ ) and condition factor ( $R^2 = 0.24$ ;  $P < 0.01$ ). Results from a two-way ANCOVA testing for the main effects of date and treatment with mass as a covariate indicated a significant effect of date ( $F_{3, 27} = 20.06$ ;  $P < 0.01$ ) and treatment ( $F_{3, 27} = 3.02$ ;  $P = 0.047$ ). The covariate mass was significant also ( $F_{1, 27} = 7.61$ ;  $P = 0.011$ ). There was no significant interaction between treatment and date ( $F_{9, 27} = 1.47$ ;  $P = 0.208$ ).

Daily ATPase activity levels increased earlier in the high and medium treatments compared with the control (Figure 5). This is seen as the difference in peak height between the control, medium, and high treatment's daily rate ATPase activity levels (0.027 vs. 0.070, 0.092  $\mu\text{mol ADP} \cdot \text{mg protein}^{-1} \text{hr}^{-1}$ ). The daily rate of change in ATPase activity in the high treatment also began to decrease earlier than in the controls.

### *3.3 96-h Seawater Adaptability Test*

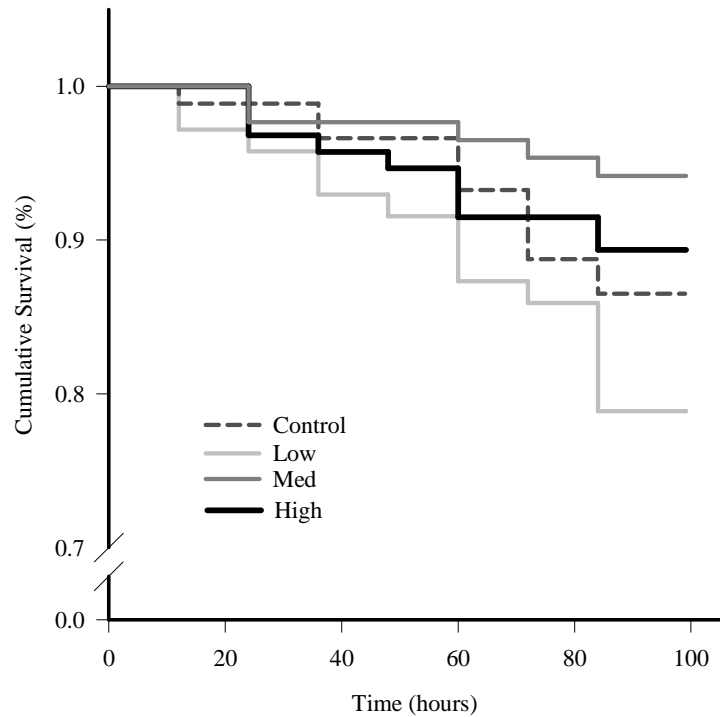
All control and treatment salmon underwent a 96 h seawater adaptability test on June 15. Twelve percent ( $n = 42$ ) of all fish (control and treatment;  $n = 340$ ) expired during the transition from freshwater to seawater (Figure 6).



**Figure 5.** The average daily rate of change in gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity ( $\mu\text{mol ADP} \cdot \text{mg protein}^{-1} \text{ hr}^{-1}$ ) from April thru June.

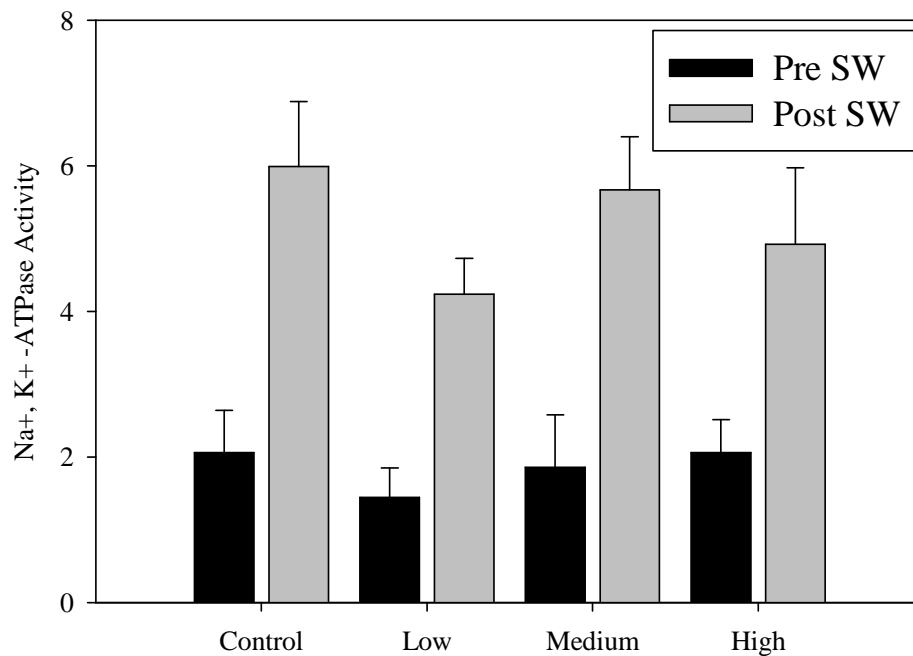
The medium treatments had the lowest mortality rate among all fish in the study (1.5%), followed by the high (2.9%), control (3.5%), and low treatment (4.4%). Mean weight for fish that survived the 96 h seawater adaptability test was  $7.86 \pm 1.94$  g ( $n = 298$ ) whereas mean weight for individuals that expired was  $4.87 \pm 1.69$  g ( $n = 42$ ).

Results from a Cox proportional hazard test indicated that mass had a strong influence on survival during the 96 h seawater adaptability test ( $\beta = 0.403$ ;  $P < 0.05$ ).



**Figure 6.** Cumulative survival curves for Chinook salmon during a 96 h seawater adaptability test.

The odds ratio ( $\beta$ ), defined here as the predicted change in the hazard for a unit increase in mass, indicated fish weighing greater than 5.0 g had a 99% chance of survival during the 96 h seawater test. Condition factor (K) and fork length were not significant components to survival and were removed from the model. There was no effect of treatment on survival. Weight was the most influential factor related to survival in the 96-hour seawater adaptability test.



**Figure 7.**  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity pre and post 96 h seawater exposure in control and treatment groups fed dietary methylmercury. Error bars are  $\pm 1$  S.E.M.

The juvenile salmon were analyzed using a one-way ANOVA to determine if there were differences among treatments in gill ATPase activity following seawater exposure (Figure 7). Results indicated there was no significant effect of diet ( $F_{3,8} = 25.85$ ;  $P = 0.054$ ). As expected there was a noticeable increase in ATPase activity in all treatments following seawater exposure (Figure 7). Table 1 summarizes the mean fork length, weight, and condition factor (K) for pre and post seawater salmon.



**Table 1.** Fork length, weight, and condition factor (K) post seawater challenge in control and treatments salmon fed dietary methylmercury (mean  $\pm$  SD). \*The Number of individual salmon at the start of the seawater adaptability test on June 16<sup>th</sup>, 2008.

<b>Group</b>	<b>Replicate</b>	<b>final/initial*</b>	<b>F.L. (mm)</b>	<b>Mass (g)</b>	<b>Condition Factor (K)</b>
<b>Control</b>	1	25/29	96.8 $\pm$ 7.4	8.3 $\pm$ 2.1	0.90 $\pm$ 0.07
	2	24/30	94.4 $\pm$ 7.0	7.0 $\pm$ 1.8	0.82 $\pm$ 0.07
	3	28/30	96.9 $\pm$ 6.5	8.0 $\pm$ 1.6	0.87 $\pm$ 0.08
	Expired	12	80.3 $\pm$ 8.3	5.3 $\pm$ 1.5	0.98 $\pm$ 0.05
		<b>77</b>	<b>96 <math>\pm</math> 6.9</b>	<b>7.8 <math>\pm</math> 1.9</b>	<b>0.86 <math>\pm</math> 0.08</b>
<b>Low</b>	1	13/21	93.9 $\pm$ 7.2	7.3 $\pm$ 1.9	0.86 $\pm$ 0.10
	2	17/23	97.4 $\pm$ 9.1	8.5 $\pm$ 3.0	0.89 $\pm$ 0.09
	3	26/27	94.2 $\pm$ 6.3	7.4 $\pm$ 1.8	0.87 $\pm$ 0.07
	Expired	15	81.5 $\pm$ 9.0	5.2 $\pm$ 1.6	0.93 $\pm$ 0.09
		<b>56</b>	<b>95 <math>\pm</math> 7.5</b>	<b>7.7 <math>\pm</math> 2.3</b>	<b>0.87 <math>\pm</math> 0.08</b>
<b>Medium</b>	1	23/24	96.5 $\pm$ 6.4	8.2 $\pm$ 1.9	0.90 $\pm$ 0.08
	2	30/33	94.6 $\pm$ 4.7	7.3 $\pm$ 1.4	0.86 $\pm$ 0.09
	3	28/29	95.9 $\pm$ 7.3	7.7 $\pm$ 2.0	0.86 $\pm$ 0.07
	Expired	5	78.6 $\pm$ 13.0	4.9 $\pm$ 2.0	0.96 $\pm$ 0.02
		<b>81</b>	<b>95.6 <math>\pm</math> 6.1</b>	<b>7.7 <math>\pm</math> 1.8</b>	<b>0.87 <math>\pm</math> 0.08</b>
<b>High</b>	1	27/31	96.4 $\pm$ 7.8	8.4 $\pm$ 1.8	0.93 $\pm$ 0.09
	2	31/35	96.3 $\pm$ 9.1	7.9 $\pm$ 2.3	0.86 $\pm$ 0.10
	3	26/28	97.2 $\pm$ 4.9	8.3 $\pm$ 1.5	0.89 $\pm$ 0.08
	Expired	10	72.9 $\pm$ 11.4	4.0 $\pm$ 1.8	0.94 $\pm$ 0.07
		<b>84</b>	<b>96.7 <math>\pm</math> 7.5</b>	<b>8.2 <math>\pm</math> 1.9</b>	<b>0.89 <math>\pm</math> 0.09</b>

#### 4.0 Discussion

The characteristics of the rearing environment are important to juvenile salmonids (Shrimpton et al., 2007). Seasonally flooded wetlands and marshes especially enhance salmon growth and survival in California's SSJR Delta (Sommer et al., 2001). Wetlands and marshes offer a number of advantages over the faster moving less productive waters of a major river. Microhabitats provide salmon protection from predators and increased primary production leads to a greater abundance of food such as phytoplankton and small invertebrates. Unfortunately, in the SSJR, these types of environments are associated with greater rates of MeHg production (Heim et al., 2007) and elevated MeHg concentrations in fish and invertebrates (Ackerman and Eagles-Smith, 2010; Ackerman et al., 2010; Slotton et al., 2002). This is of serious concern for juvenile salmon as the greater growth rates indicate a higher rate of MeHg accumulation.

Juvenile salmon grow the fastest during the parr-phase of development that typically coincides with the timing of flooded wetlands in the SSJR. In Atlantic salmon (*Salmo salar*), the accumulation of MeHg was greatest in parr rather than grow out stages (Chou, 2007). This is worrisome as it suggests there may be an increased susceptibility for accumulating MeHg at a much greater rate while foraging in the SSJR wetlands. Growth rates observed in this study were also typical with the parr-smolt transition: parr grew fastest pre-smolt and growth slowed during the parr-smolt transition in late May as indicated by the elevated ATPase activity and condition factor. Juvenile salmon were estimated to have assimilated 83% of the MeHg administered over the 67 d study period. In comparison, environmental assimilation efficiencies of MeHg ranging between 85 and

94% were reported for mosquito fish (*Gambusia affinis*) and redear sunfish (*Lepomis microlophus*) collected from the SSJR Delta (Pickhardt and Fisher, 2006). The rate of MeHg assimilation in this study was not surprising considering a majority of the MeHg present in fish accumulates almost entirely through dietary uptake (Hall et al., 1997).

Juvenile salmon can spend upwards of 6 months in the SSJR Delta and possibly over-winter depending on the type of “run” and environmental conditions at the time of their smolting. The estimated assimilation efficiency from this study, albeit slightly less than what has been observed in field studies, indicates suggests they are susceptible to incorporating large amounts of MeHg while in the SSJR and even in a relatively short period. Henery et al.(2010) reported juvenile free-ranging salmon in the Yolo Bypass floodplain (a large seasonally flooded wetland in the SSJR) had increased MeHg tissue concentrations as a much as 5% per day in salmon which residing on the floodplain from 5-55 days. The range of MeHg concentrations reported in the Yolo floodplain was within the control and low treatment groups from this experiment (0.018 – 0.234  $\mu\text{g MeHg g}^{-1}$ ).

Growth is paramount to survival and seawater adaptability in juvenile Chinook, and size is a critical component in seawater tolerance. In this study, there were no significant differences in mass, length, or condition factor among dietary treatments. Similar experimental results have been observed in Atlantic salmon fed much greater contaminated diets of  $\text{HgCl}_2$  (0, 10, 100,  $\text{mg Hg Kg}^{-1} \text{ dw}$ ) and  $\text{MeHgCl}_2$  (0, 5, 10  $\text{mg MeHg kg}^{-1} \text{ dw}$ ; Berntssen et al., 2003).

The effects of dietary MeHg on growth in other species of fish were somewhat ambiguous. Reduced growth (mass and length) have been reported in male juvenile walleye (*Sander vitreus*) fed a contaminated diet of 1  $\mu\text{g MeHg g}^{-1}$  (Friedmann et al., 1996; Munn and Short, 1997). Dietary MeHg had no effect on growth in juvenile fathead minnows (*Pimephales promelas*; Drevnick and Sandheinrich, 2003), nor on the mass of adult killifish, though mortality was significantly increased in the male killifish (*Fundulus heteroclitus*) exposed to concentrations greater than 0.5 mg MeHg Kg<sup>-1</sup> (Matta et al., 2001).

In contrast to manipulative studies, an inverse relationship has been reported between MeHg concentrations and condition factor for several species of “wild” fish (Drevnick and Sandheinrich, 2003; Munn and Short, 1997). Hence, the sub lethal effect MeHg has on growth may be more profound in the wild where the effect of MeHg alters foraging behavior. Methylmercury can severely impair the fish’s ability to locate and capture prey and avoid predation (Grippo and Heath, 2003; Sandheinrich and Weiner, 2010; Smith and Weis, 1997). Manipulative studies can bias growth effects by providing ample amounts of food (Wiener and Spry, 1996). This may account for the lack of significant size differences among treatments observed in this study as all of the fish were fed to satiation daily and there was little competition for food.

Changes in ATPase activity are associated with the process of smoltification in juvenile salmon. Environmental stress (photoperiod, temperature, water flow, food availability, etc.) affects the timing of smoltification and subsequent ATPase activity in

salmon. Stressors, via cortisol and other signaling hormones, set in motion interactions between endocrine systems responsible for osmoregulation (Björnsson et al., 2011). They are “synergistic” in so much as any single stressor, alone, does not elicit as great a change in duration and timing of ATPase activity as multiple stressors acting in conjunction. Similar to an orchestra, the salmon’s environment produces a multitude of sound making the process of smoltification the symphony of physiological, chemical, and behavioral change. Increases in ATPase activity are an indication of the parr-smolt transformation and that the juvenile salmon are capable of migrating seaward. In this study, the timing of peak ATPase activity in the medium and high MeHg diets occurred earlier than observed in control treatments and duration of peak activity was longer compared to the control diets (no MeHg). Whereas MeHg may have caused an early and significant elevation in ATPase activity in the medium and high treatments, other factors, such as photoperiod and size, were important in keeping activities elevated in this study. These results suggest that differences in ATPase activity observed in this study stemmed from altered cortisol levels.

Growth hormone and cortisol, separately and in conjunction, are shown to elevate ATPase activity in the gills of Atlantic salmon (Pelis and McCormick, 2001). Chronic Hg exposure leads to an exhaustion of cortisol because of prolonged activity of the system (Hontela et al., 1992). The effect cortisol has on ATPase activity though, will vary depending on duration, concentration, and administration. For instance, cortisol levels were altered in Rainbow trout (*Oncorhynchus mykiss*) following acute aqueous Hg exposure (Bleau et al., 1996) and cadmium exposure (Tort et al., 1996). Similarly,

ATPase activity and cortisol levels increased in common carp during short term copper exposures (De Boeck et al., 2001). Sodium and chloride levels were observed to increase in Brook trout exposed to low concentrations of aqueous MeHg (Christensen et al., 1977), which was indicative of an increase in ATPase activity, though, cortisol was not measured.

ATPase activity, as an index for physiological condition, alone does not constitute the whole effect Hg has on the juvenile salmon. However, it does reflect the metabolic energy status of a particular system. In Chinook salmon, ATPase activity is related to their seawater readiness and a propensity to migrate to sea. Therefore, an argument can be made that exposure to dietary MeHg may induce earlier than expected emigration in wild juvenile Chinook salmon. This would be detrimental to Chinook as size is important for successful transition to the saltwater environment.

Mass was the most significant factor for determining survival in the seawater challenge. Individuals greater than 5 g had a greater chance of survival during the 96-h seawater exposure than did individuals that were less than 5 g. The low diet group experienced the greatest amount of mortality during the 96 h seawater adaptability test. One explanation for the decrease in survivability in this treatment may be the dose of MeHg administered.

Other researchers have indicated low or mid-level doses of Hg can greatly reduce survival in fish without those same profound effects on the higher level treatments (Wiener et al., 2003). One possible cause is chronic low level exposures of Hg may

cause the organism to use a greater amount of energy, which could have been put towards growth, but instead was used to de-toxify the system. At greater levels of Hg, the system is overwhelmed, and the internal defense system turns off (e.g. cortisol levels become exhausted). In this scenario, less energy is used producing de-toxifying proteins and more energy is put towards somatic growth.

Post seawater differences among treatment and control ATPase activity were not significant ( $P = 0.054$ ). However, all control and treatment groups were elevated above freshwater activities as expected with the introduction to seawater (ATPase activity not measured during the seawater challenge). The control group had the greatest response in ATPase activity. However, ATPase activity in the low MeHg treatment responded the least compared with its freshwater activity. This group also had the greatest mortality associated with the experiment as previously mentioned. This further substantiates the hypothesis that the low dietary group did not have the energy necessary to acclimate to an increase in salinity. Instead, the energy was put towards detoxifying the system.

## **5.0 Conclusion**

In this study, there were three measures used to test the effects of dietary MeHg on juvenile Chinook salmon: Size (mass, length, and condition factor), ATPase activity, and seawater adaptability. The timing of peak ATPase activity in the medium and high MeHg groups occurred significantly earlier and peak activity was greater in duration than observed in controls. Results from the seawater adaptability test indicated, in all treatments, that size (mass) was the most significant variable related to survival.

Overall, the findings from this study indicate MeHg can induce subtle changes in juvenile salmon physiology, even at relatively low levels. This study did not examine the behavioral effects of MeHg at these sub-acute levels, yet MeHg is known to produce negative neurological effects. If such an effect is manifest, such as the smolts ability to avoid predation or navigate to its home region, significant impacts to the population could result. Such a sub acute behavioral response was recently shown to be lethal to bee colonies (*Apis mellifera*), when physiological impacts at low exposure levels were not observed (Henry et al., 2012). In the wild, the physiological changes in ATPase activity caused by MeHg exposure also may be indicative of behavioral changes considering cortisol is closely linked to ATPase activation. In the SSJR, behavioral changes might lead to reduced foraging capability or an early migration to sea.

Reductions in habitat, water quality, and over-fishing have negatively affected one of the historically largest populations of Chinook salmon along its range (Yoshiyama et al., 1998). Proposals to create new wetlands in the SSJR by flooding existing agricultural fields will create beneficial habitat and enhance growth in the Chinook salmon. The extent to which increased production of MeHg from these wetlands will have on Chinook smoltification physiology and development should be of concern.



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